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Avian influenza vaccines and therapies for poultry

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Abstract

Vaccines have been used in avian influenza (AI) control programs to prevent, manage or eradicate AI from poultry and other birds. The best protection is produced from the humoral response against the hemagglutinin (HA) protein. A variety of vaccines have been developed and tested under experimental conditions with a few receiving licensure and field use following demonstration of purity, safety, efficacy and potency. Current licensed vaccines are predominately inactivated whole AI vaccines, typically produced from low pathogenicity (LP) AI virus strains, or occasionally from high pathogenicity AI virus strains. Recently, reverse genetic procedures have been developed that allow construction of vaccine strains using a genetically altered HA gene (changing HP HA proteolytic cleavage site to LP) and a backbone of internal gene segments for safe, high growth production. Other licensed AI vaccines include recombinant fowl poxvirus vector with an AI H5 insert and a recombinant Newcastle disease virus vector with an AI H5 gene insert. The latter vaccine can be mass administered via aerosol application.
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Keywords: Avian influenza; Vaccine; Protection; Efficacy; Potency

Résumé

Lors des programmes de contrôle de la grippe aviaire, des vaccins ont été utilisés pour prévenir, contrôler ou éradiquer le virus chez les volailles et autres oiseaux. Seule une réaction humorale à l'hémagglutinine (HA) permet d'obtenir une bonne protection. Parmi la gamme de vaccins développée et testée à titre expérimental, seuls quelques-uns ont reçu une licence et un champ après avoir démontré leur pureté, leur sûreté, leur efficacité et leur puissance. Les vaccins possédant actuellement une licence sont majoritairement des vaccins contre la grippe aviaire non actifs,

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généralement produits à partir de souches faiblement pathogènes, ou plus rarement à partir de souches virales hautement pathogènes. Récemment, des procédures génétiques inversées ont été développées afin de permettre la fabrication de gènes hautement pathogènes génétiquement modifiés (en changeant le site de division protéolytique de l'hémagglutinine hautement pathogène en faiblement pathogène) et d'une structure de segments génétiques internes pour une production massive et sûre. D'autres vaccins sous licence comprennent un vecteur du virus de la variole aviaire recombiné avec la grippe aviaire H5 et un virus de la maladie de Newcastle recombiné avec un gène de la grippe aviaire H5. Ce dernier vaccin peut être administré en masse par aérosol.
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Mots clés : grippe aviaire ; vaccin ; protection ; efficacité ; puissance

1. Introduction

Avian influenza (AI) is an infection of birds caused by a single stranded, negative-sense RNA virus in the family Orthomyxoviridae, genus influenzavirus A (influenza A virus) with the genome divided into eight gene segments. Influenza A virus are further categorized by serological reaction of the two surface glycoproteins into 16 different hemagglutinin (H1–16) and 9 different neuraminidase (N1–9) subtypes [1]. Protection is primarily the result of humoral immune response against the hemagglutinin (HA), and secondarily against the neuraminidase, but such protective responses are only subtype specific; i.e. there is no single AI vaccine that is protective against all subtypes. Avian influenza (AI) viruses vary in virulence either being of low (LP) or high pathogenicity (HP).

2. Control programs

Control programs for AI are designed to achieve one of three broad goals or outcomes: (1) prevention, (2) management, or (3) eradication [2,3]. The individual goal or outcome is achieved through incorporating various essential components including inclusion and exclusion biosecurity practices, diagnostics and surveillance, elimination of infected animals, increasing host resistance, and education of personnel in AI control strategies. The level of incorporation and practice of these five components will determine whether the control strategy will prevent, manage or eradicate AI. Vaccines can be used as a single tool in a comprehensive strategy by increasing host resistance to AI virus infection and decreasing environmental contamination, but other components are essential in order to achieve success within any of these three goals. Practice of AI vaccination varies around the world with infrequent use of vaccines in the developed world to some developing countries routinely using AI vaccines for control of H9N2 (Asia and Middle East) and H5N2 (Central America) LPAI, and H5N1 HPAI (Asia and Africa). However, vaccines have not been a universal solution in the control of AI in the field. Concerns have been raised about inconsistencies in field protection with quality of some vaccines and inadequate administration being issues.

3. Avian influenza vaccine technologies

Based on experimental studies and field usage, influenza A virus vaccines can be categorized into four broad technological groups: (1) inactivated whole influenza viruses, (2) *in vitro* expressed HA protein, and (3) *in vivo* expressed HA protein, and (4) nucleic acid vaccines.

First, LPAI viruses (LPAIV) isolated from outbreaks in poultry or from surveillance in wild or domesticated birds have been the primary virus strains used in inactivated AI vaccines—a mature, low cost technology used for over the past 30 years. Typically, these viruses have been grown in 9–11-day-old embryonating chicken eggs, the infected allantoic fluid harvested, the virus inactivated using chemicals (such as formalin, β-propiolactone or binary ethylenimine), and the crude preparation or purified hemagglutinin emulsification in oils and surfactants. In a few situations, H7N3 in Pakistan and H5N1 in Indonesia, HPAI strains have been used as virus strains in inactivated AI vaccines. Today, with the development of reverse genetic systems for AI virus [4,5], custom made inactivated AI vaccines are possible using these infectious clones systems [6,7]. These viruses can be tailored to very close genetic similarity to field viruses and thus close antigenic similarity, but be rendered safe by altering the HA cleavage site from a HP to LPAI virus. In addition, the six genes that encode the internal proteins can be derived from an existing influenza A virus vaccine strain thus imparting properties of high virus growth titers in embryonating eggs, and thus resulting in the incorporation of high HA antigen content in the vaccine at low cost. AI vaccines using the oil adjuvant system have the advantage of producing very high antibody titers in poultry and the protection from homologous HA field strains is good over extended periods of time [8]. However, usage of inactivated AI vaccines is limited because of high labor cost for parenteral administration of the vaccines and difficulty in identifying infected birds by routine serological tests in the vaccinated population; i.e. differentiating infected from vaccinated animals (DIVA). Special serological tests need to be developed for DIVA strategy. Finally, protective response is very dependent on antigen quantity in each dose and adjuvant system.

Second, large quantities of HA can be expressed in an *in vitro* system and this crude or purified HA can be incorporated into the vaccine. This can be accomplished by inserting the HA gene into eukaryotic cell cultures, plants, yeast [9], bacterial (e.g. *Escherichia coli* [10]), or viral vectors (e.g. baculovirus [11]), and usage of industrial methods to produce the AI viral HA, followed by oil emulsification. This makes the vaccine technically similar to inactivated whole AI vaccines, but the HA antigen is produced in an *in vitro* system without safety concerns of growing AI vaccine viruses.

Third, the protective antigens can be expressed *in vivo* such as using live administered bacterial or viral vectors with nucleic acid inserts of HA gene as either cDNA or RNA. These vectored vaccines have included fowl poxvirus [12], vaccinia virus [13], Rous sarcoma virus [14], adenoviruses [15,16], Venzuelan equine encephalitis virus [17], Newcastle disease virus [18–20], herpesvirus of infectious laryngotracheitis [21], and an avian influenza-Newcastle disease virus chimera [20]. At this time, no live AI virus vaccines are available for use in animals, but cold adapted temperature sensitive mutants influenza A virus vaccines has been developed and are safe for use in humans [22,23]. Potentially, a live AI virus vaccine could be developed for use in poultry if adequate safety were demonstrated.

The recombinant fowl poxvirus-AI-H5 (rFP-AIV-H5) has been the most common vectored technology used in licensed AI vaccines. The rFP-AIV-H5 have some advantages over inactivated AI vaccines: (1) they are administered in the hatchery which improves the biosecurity and quality control of vaccination and (2) DIVA strategies are easy to develop because they use existing serological tests to detect antibodies against vaccine HA (HI) and lack of antibodies to NP (agar gel immunodiffusion [AGID] or ELISA test) present in AI virus infected birds. However, the rFP-AIV-H5 has some limitations: (1) rFP can only be used in chickens, and (2) chickens must be naive to the FP vector for immunization to be effective. Also, like inactivated AI vaccines, parenteral injection is required. However, the recombinant Newcastle disease virus-AI with H5 or H7 AI virus gene inserts has advantages over both (1) and (2) above by allowing administered by mass application (aerosol spray or eye drop) thus reducing the cost of administration, protects against subtype specific AIV and all NDV, and uses existing serological tests as a DIVA serological strategy. However, preexisting maternal and active humoral immunity to ND virus vector or AI virus may limit vaccine replication and thus reduce or prevent a protective immune response. Finally, safety of such live vaccines must be assessed to ensure environmental protection.

Fourth, nucleic acid vaccines, primarily as DNA plasmids with cDNA inserts that code for HA, provide protection via uptake and expression by antigen-presenting cells or myocytes and ensuing immune response [24]. Influenza A virus HA-based DNA vaccines are possible, but with current technologies, the cost is prohibitive [24,25]. Additional research needs to be done to improve adjuvants and identify better promoters for poultry [3].

4. Avian influenza vaccines in use

The frequency and quantity of AI vaccine used in poultry has been poorly documented throughout the world until the last 20 years, but has been of low usage globally until the last decade. Historically, AI vaccines have been used most frequently in control and eradication programs for LPAI viruses in small geographic areas of high risk; e.g., in the USA, the most frequent use of influenza A virus vaccines have been to protect turkey breeders against H1N1, H1N2 and H3N2 swine influenza viruses. During 2001, 2.6 million doses of inactivated vaccine against H1 swine influenza was used in turkey breeders in the USA [26]. In another example, historically waterfowl-origin LPAI viruses have been known to infect meat turkeys raised under outdoor range conditions in Minnesota during the fall wild duck migration. As a response to such risk, from 1978 to 1996, 22 million doses of inactivated AI vaccines were used in Minnesota but moving production in door has eliminated seasonal LPAI outbreaks [27,28]. Other examples of more recent and high usage of inactivated AI vaccine has been in northern Italy against H7N1 and H7N3 LPAI virus in layers and turkeys (2000–2006), and H9N2 LPAI virus in the Middle East and Asia (late 1990s–present) in layers and broilers.

Use of AI vaccines in control programs for HPAI has been historically infrequent until recently. The first usage of AI vaccine in HPAI outbreak was in Mexico during the H5N2 HPAI virus (HPAIV) in 1995. However, AI vaccine has continued to be used in controlling

H5N2 LPAI in Mexico, Guatemala and El Salvador (1995–2006) with 1.8 billion doses of H5N2 inactivated vaccine and 2 billion doses recombinant fowlpox-AIV-H5 vaccine used since 1995. In Pakistan, in response to H7N3 HPAIV, inactivated H7N3 vaccine was used in 1995 with expanded usage when H7N3 HPAI occurred in two other regions of Pakistan (2001 and 2004). The emergence of H5N1 in Asia has lead to usage of inactivated H5N2 vaccine in Hong Kong poultry (2002–2006), followed by H5 vaccine usage in Indonesia (2003), China (2004), India (2006), Pakistan (2006), Vietnam (2005), Russia (2005) and Egypt (2006). For China alone, from January 2004 through December 2006, 22.7 billion doses of H5 AI vaccine has been used, mostly inactivated H5, but also some recombinant fowl poxvirus and recombinant Newcastle disease virus with AI H5 gene inserts [29]. Finally, following an outbreak of H7N7 in North Korea, inactivated vaccine was used in 2005.

In the H5N1 HPAI outbreaks, a variety of vaccine technologies and virus strains have been used in the field. The majority of vaccines have been inactivated whole AI virus vaccines utilizing AI outbreak viruses in manufacturing: A/turkey/England/73 (H5N2) LPAIV, A/chicken/Mexico/94 (H5N2) LPAIV, A/chicken/Indonesia (Legok)/03 (H5N1) HPAIV, and A/turkey/Wisconsin/68 (H5N9) LPAIV. Recently, two reverse genetic produced vaccines have been developed and used in traditional oil emulsified inactivated AI vaccines: (1) H5 and N1 genes of A/goose/Guangdong/96 with six internal genes from PR8 influenza A virus vaccine strain [30] and (2) H5 gene from A/chicken/Vietnam/c58/04 (H5N1), N3 gene from A/Duck/Germany/1215/73 (H2N3) and six internal genes from PR8 strain [7]. Recombinant fowl poxvirus with cDNA inserts of AI viral genes were first used in Mexico in 1997, but in last 2 years rFP-AIV-H5 vaccines have been used in Asia against H5N1 HPAIV. These rFP-AIV have cDNA inserts of the H5 gene from A/turkey/Ireland/83 (H5N9), or the H5 and N1 genes from A/goose/Guangdong/96 (H5N1). In both situations, the HA gene was altered from a HPAIV to a LPAIV. Finally, recombinant Newcastle disease vectors with different AIV H5 gene inserts have been developed and are licensed for use in China and Mexico [29,31].

5. Features of vaccines for licensing

For a veterinary vaccine to be licensed for use, the vaccine should be shown to be: (1) pure, i.e. not adulterated, containing only the desired compounds and must be consistent in composition; (2) safe, i.e. no deleterious effect on vaccinated host or environment; (3) efficacious, i.e. meets specific quantifiable protection criteria; and (4) potent; i.e. sufficient antigen mass or dose to ensure efficacious under a variety of conditions.

6. Assessment of vaccine efficacy

6.1. Direct assessment

The assessment of a protective immune response by AI vaccines is best accomplished using the ‘‘Gold Standard’’, a LP or HPAIV challenge model. Protection can be quantified

Table 1
Vaccine protection as measured by prevention of clinical signs (morbidity) and death (mortality)

Vaccine group	Challenge virus	Morbidity	Mortality (mean death time)	Survivors (%)
rB1H7, 1x	Fontana	0/10	0/10	100
rB1H7, 1x	Steele	1/10	1/10 (4.0)	90
rB1H7, 2x	Fontana	0/10	0/10	100
rB1H7, 2x	Steele	1/10	1/10 (7.0)	90
SEP B1, 2x	Fontana	0/10	0/10	100
SEP B1, 2x	Steele	10/10	10/10 (3.3)	0
Sham, 2x	Fontana	10/10	10/10 (4.0)	0
Sham, 2x	Steele	7/10	7/10 (2.3)	30

Chickens vaccinated at 2 and 4 weeks-of-age with recombinant NDV with H7 AI gene insert or B1 parent NDV vaccines (eye drop), and intranasally challenged at 6 weeks-of-age with 10^5 EID_{50} of velogenic viscerotrophic NDV (aPMV1/Fontana/73) or HPAIV (A/Steele/59 [H7N7]) [20].

Table 2
Vaccine protection as measured by reduction of challenge virus replication in respiratory and gastrointestinal tracts

Vaccine group	Virus isolation, 2 days post-challenge (log_{10} EID_{50}/ml)	
	Oropharynx[a]	Cloacal[a]
Sham	$10/10^{A}(6.6^{a})$	$10/10^{A}(5.82^{a})$
1994 H5N2 Mexican strain	$5/10^{BC}(1.23^{b})$	$3/10^{B}(1.00^{b})$
1986 H5N2 Eurasian strain	$6/10^{AC}(1.78^{b})$	$3/10^{B}(1.53^{b})$

Chickens were vaccinated subcutaneously at 3 weeks-of-age with one of two inactivated whole AI virus vaccines and intranasally challenged 3 weeks later with high dose HPAIV ($10^{6.0}$ EID_{50} A/chicken/Indonesia/7/2003 [H5N1]) [38]. Swabs for virus isolation and titration were taken at peak of replication, i.e. 2 days post-challenge.

[a] Number of virus positive/total birds, different uppercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test; () = mean titer in log_{10} EID_{50}, different lowercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test.

by measuring prevention of clinical signs and death (HPAIV challenge) (Tables 1 and 2) [32], prevention of egg production drops (LP and HPAIV challenge) [32,33], quantitative reduction in shedding of challenge virus from respiratory and gastrointestinal tracts (LP and HPAIV challenge) (Table 2) [8,34], and prevention of contact transmission (LP and HPAIV challenge) (Table 3) [34]. Reductions in challenge virus replication and titration can be assessed by virus isolation in embryonating chicken eggs or tissue culture systems, by quantitative RRT-PCR or quantitative antigen detection. Prevention of contact transmission is a desirable goal, but the laboratory assessment is typically not standardized because multiple variables impacted outcome such as bird density, and sanitation and ventilation standards.

Ideally, efficacious AI vaccines have additional attributes including: (1) protect against high dose challenge (Table 4 and Fig. 1) [34]; (2) long periods of protection, usually a minimum of 6–12 months [34]; (3) a defined route of administration which is reproducible such as subcutaneous injection, wing web administration, coarse or fine spray, eye drop, *in ovo*, etc.; (4) a minimum number of vaccinations to achieve protection, ideally one but with some species (e.g. turkeys) and long live birds (e.g. layers), may require multiple vaccinations; and (5) protection in multiple species of birds. Because AI field viruses

Table 3
Protection from contact transmission as measured by morbidity, mortality and reduction in infection rates of *in contact* chickens [34]

Challenge group	Contact group	Morbidity	Mortality	Virus isolation from swabs (6 days post-contact)	
				Oropharynx	Cloaca
rFP-H5	rFP-H5	0/15	0/15	0/15	0/15
	cFP	0/15	0/15	0/15	0/15
cFP	rFP-H5	0/15	0/15	4/15	0/15
	cFP	6/15	4/15	3/13	2/13

Chicks were vaccinated with recombinant fowl poxvirus with H5 AI gene insert (rFP-H5) or control fowl poxvirus (cFP) vaccines at 1 day of age. At 3 weeks, one half of the birds were challenged by intranasal inoculation with 10^6 EID_{50} of HPAIV (A/chicken/Queretaro/14588-19/1995 [H5N2]). Two days post-challenge, the intranasally inoculated chickens were placed in contact with naïve vaccinated birds. In the intranasal challenged groups, rFP-H5 vaccinated group lacked morbidity (0/30) and lacked mortality (0/30) while most of the cFP group became ill (30/30) and died (24/30). The results of *in contact* birds are reported in the table above.

Table 4
Variations in prevention of clinical signs (morbidity) and death (mortality) of vaccinated chickens following challenge with different doses of HPAIV (mean embryo infectious [EID_{50}] and mean chicken lethal doses [CLD_{50}])

Vaccine	Challenge dose		Morbidity	Mortality
	EID_{50}	CLD_{50}		
rFP-H5	0.5	0.003	0/10	0/10
	2.0	0.1	0/10	0/10
	3.5	3.2	0/10	0/10
	5.0	100	0/10	0/10
	6.5	3,200	0/10	0/10
	8.0	100,000	2/10	2/10 (4.5)
Sham	0.5	0.003	0/10	0/10
	2.0	0.1	0/10	0/10
	3.5	3.2	8/10	8/10 (2.75)
	5.0	100	10/10	10/10 (2.4)
	6.5	3,200	10/10	10/10 (2.0)
	8.0	100,000	10/10	10/10 (2.0)

Chickens were vaccinated subcutaneously at 1-day-of-age with recombinant fowl poxvirus containing H5 AI virus gene insert and intranasally challenged at 3 weeks-of-age with various challenge doses of HPAIV ($10^{0.5–8.0}$ EID_{50}, A/chicken/South Korea/2003 [H5N1]) [49].

undergo antigen drift with loss of protection [35], vaccines should be re-evaluated for *in vivo* protection against current circulating field viruses every 2–3 years. However, poultry AI vaccines have had a life of usage without changing vaccine strains as compared to human influenza vaccines; i.e. one study has shown the efficaciousness for a single 1983 H5 vaccine to protect chickens against diverse H5 HPAI challenge viruses from 1959 to 1997 [36].

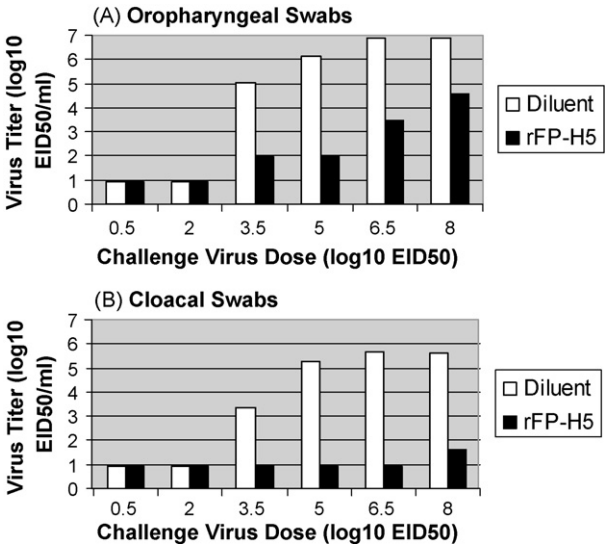

Fig. 1. Variations in reduction of oropharyngeal (A) and cloacal (B) virus shedding rates in vaccinated chickens following challenge with different doses of HPAIV. Chickens were vaccinated subcutaneously at 1-day-of-age with recombinant fowl poxvirus containing H5 AI virus gene insert (rFP-H5) or diluent, and intranasally challenged at 3 weeks-of-age with various challenge doses ($10^{0.5–8.0}$ EID_{50} of HPAIV A/chicken/South Korea/2003 [H5N1]) [49]. No virus was detected in any swabs from vaccinated chickens challenged with $10^{0.5}$ or $10^{2.0}$ challenge doses or cloacal swabs of rFP-H5 chickens challenged with $10^{3.5–6.5}$ EID_{50}.

6.2. Indirect assessment

Direct assessment of efficacy is time consuming and expensive, but necessary in initial demonstration of vaccine efficacy. Indirect assessment is a viable approach for assuring consistency of vaccine batches at a minimal protection level. Indirect assessment can be based on serological responses in vaccinated birds where it is based on serological titers that correlate with *in vivo* protection such as neutralization or hemagglutination inhibition (HI) titers (Table 5). Another indirect assessment is quantification of hemagglutinin protein in

Table 5
Indirect assessment of vaccine protection from mortality by measuring serological response

Vaccine group	Mortality	Pre-challenge serology	
		AGID	HI(GMT)
Sham	10/10	0/10	0/10 (<8)
1994 H5N2 Mexican strain	0/10	10/10	10/10 (128)
1986 H5N2 Eurasian strain	1/10	9/10	9/10 (120)

Chickens were vaccinated subcutaneously at 3 weeks-of-age with one of two inactivated whole AI virus vaccines and intranasally challenged 3 weeks later with high dose of HPAIV ($10^{6.0}$ EID_{50}, A/chicken/Indonesia/7/2003 [H5N1]) [38]. HI antibody results correlate with protection from a challenge virus of the same HA subtype. However, the presence of AGID antibodies (against nucleoprotein) is not predictive of protection except for challenge viruses of the same HA subtype as vaccine the virus.

Table 6
Indirect assessment of vaccine protection (mortality and challenge virus shedding) by using HA protein quantity and HI serology

Dose (μg)	HI[a] (log 2)	Mortality[b]	Virus isolation 3 days post-challenge[c] (EID_{50}/ml)	
			Oral	Cloacal
0.005	$0/7^{a}(0)$	$7/7^{a}$	$5/5^{A}(5.6^{a})$	$2/5^{A}(1.1^{a})$
0.05	$5/7^{b}(8)$	$2/7^{b}$	$7/7^{A}(5.5^{ab})$	$2/7^{A}(1.4^{a})$
0.5	$7/7^{b}(8)$	$0/7^{b}$	$7/7^{A}(4.6^{b})$	$0/7^{A}(<0.9^{b})$
5	$7/7^{b}(8.5)$	$0/7^{b}$	$7/7^{A}(3.8^{b})$	$0/7^{A}(<0.9^{b})$

Chickens were vaccinated subcutaneously at 4 weeks-of-age with inactivated whole AI vaccine (A/chicken/Pueblo/28159-474/1995 [H5N2]) and intranasally challenged at 7 weeks-of-age with high challenge dose of HPAIV (10^6 EID_{50} A/chicken/Queretaro/14588-19/1995 [H5N2]) [8]. Different superscript letter indicate statistical differences ($P < 0.05$) within the column.

[a] Number of HI positive/total birds, different lowercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test.

[b] Number of dead/total birds, different uppercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test.

[c] Number of virus positive/total birds, different uppercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test; () = mean titer in $\log_{10}$ EID_{50}, different lowercase superscript letter indicate statistical differences ($P < 0.05$) within the column using Fisher's Exact test.

inactivated AI vaccine or infectious titer of live vaccines (Table 6). The amount of antigen in inactivated vaccines can be assessed by radial immunodiffusion assay [37], quantitative RRT-PCR assay [38], infectious titer prior to inactivation [8], hemagglutination titer [8] and other methods of hemagglutinin protein quantification. For recombinant vaccines, virus replication and titration in embryonating chicken eggs or cell cultures are appropriate.

7. Potency

Potency standards for vaccine are important to assure the vaccine will be efficacious in the field under a variety of conditions, and are usually focused on providing sufficient antigen mass or vaccine dose to ensure efficacious. Initially, the mean protective dose (PD_{50}) of the vaccine in specific pathogen free chickens under laboratory conditions is determined (Table 7). This would be followed by tests in other avian species and under different simulated field conditions. However, since the list of field variables is endless, an alternative in standardization is to require a minimum number of PD_{50} per dose which

Table 7
Comparison of PD_{50} between different H5 inactivated AI vaccines (mineral oil emulsified) and the level of HA protein to achieve 50 PD_{50} doses [8]

Vaccine strain	PD_{50} (μg viral HA)	50 PD_{50} (μg viral HA − [35 PD_{50}])
TW/68	0.006	0.3 (0.21)
TM/81	0.156	7.8 (5.46)
M5/94	0.016	0.8 (0.56)
P3/95	0.030	1.5 (1.05)

should be adequate under a variety of field conditions. For Newcastle disease vaccines, the minimum is usually set at 50 PD_{50} with a minimum dose not below 35 PD_{50} (Table 7) [39]. The PD_{50} can be produced based on protection following *in vivo* challenge studies or serological response using a method that produces titers that correlate with protection.

8. Limitations and disadvantages of avian influenza vaccines

Monitoring of vaccine impact in the field is crucial in on going evaluation of the vaccine's efficacy. Because immunity against AI virus is not absolute in the field and vaccination may not be optimal, vaccinated populations of birds can potentially become infected with field virus and shed the virus into the environment. As a result, monitoring programs must be designed to detect infections among vaccinated populations. This monitoring can be done several ways: (1) specific serological tests to detect antibodies against NS-1 protein or heterologous neuraminidase (NA or NS1) in birds vaccinated with inactivated whole AI vaccines [38,40,41]; (2) routine serological tests to detect antibodies against HA (HI) or nucleoprotein (AGID or ELISA) in birds vaccinated with *in vitro* or *in vivo* express HA vaccines or DNA vaccines, or unvaccinated sentinel birds [11]; (3) virus detection in daily mortality using virus isolation, qRRT-PCR or antigen capture [42].

9. Therapeutics for avian influenza viruses in poultry

Experimentally, amantadine, an M2 ion channel blocker, has been shown to be effective in reducing mortality in HPAI-infected poultry, but the drug is not approved for food animals, and its use rapidly gives rise to amantadine-resistant viruses [43–47]. The detection of H5N1 and H9N2 amantadine-resistant AI virus strains in poultry from China was purported to be associated with addition of amantadine in the feed by some farmers [48]. Currently, anti-influenza A virus drug therapy is not recommended for poultry and such anti-viral drugs should be used only in humans in order to minimize the development of influenza A virus resistant strains. Supportive care and antibiotic treatment have been employed to reduce the effects of concurrent bacterial infections as an aid in recover of poultry and other birds from LPAI.

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